2012

The effect of air on the potency of multi-use vials of Mycobacterium bovis purified protein derivative (bPPD)

Teresa Marie Sigafoose
Iowa State University

The effect of air on the potency of multi-use vials of *Mycobacterium bovis* purified protein derivative (bPPD)

by

Teresa Marie Sigafoose

A thesis submitted to the graduate faculty

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Immunobiology

Program of Study Committee:
Jesse M. Hostetter, Co-major Professor
W. Ray Waters, Co-major Professor
Walter Hyde

Iowa State University

Ames, Iowa

2012

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

The effects of air on the potency of human tuberculin purified protein derivative (PPD) was studied 30 years ago and based on this information, recommendations as to the bottling and handling of multi-use vials were made to manufacturers and health care workers to reduce air exposure to the PPD . Until this current study, there has been no published work done to determine the effect of air on maintaining potency of bovine PPD (bPPD). Bovine PPD is used by the United States Department of Agriculture (USDA) Tuberculosis (TB) Eradication Program as an *in vivo* diagnostic test for bovine tuberculosis. Currently, guidelines issued by the USDA TB Eradication Program call for the disposal of multi-use vials of bPPD 2 weeks after the initial use.

In this study, we utilized one lot of bPPD that was distributed to field personnel to test for bovine TB. Seven bottles of bPPD were exposed to air and held at 4°C storage conditions for varying time periods over the course of 6 months. One bottle of bPPD was never exposed to air and used as a negative control reference. Duplicates of each experimental PPD were utilized. At the end of 6 months, potency testing was performed *in vivo* in sensitized guinea pigs and experimentally infected cattle. The Bovigam® assay was used as an *in vitro* potency tool to measure gamma interferon production by lymphocytes upon stimulation utilizing bPPDs from each treatment group. Protein concentration remained the same in all bottles as well as the pH and no visual changes were apparent.

We found that bPPD retained acceptable potency for up to 6 months after being exposed to air, stored at 4 °C, and in the original amber colored multi-use vials.

CHAPTER 1. INTRODUCTION

Bovine TB is a disease caused by the bacteria *Mycobacterium bovis*. It was recognized in 1877 that cattle infected with tuberculosis could transmit the disease to humans (Miller 1989). Testing for this disease in cattle began in the United States in 1892 (Konya 1977). At that time, the formulation created by Robert Koch, Koch's tuberculin, composed of *Mycobacterium tuberculosis* cell culture lysates and glycerine was used to test both cattle and humans (Gradmann 2006). Since that time, there have been advances in the field of bovine TB diagnostics that include: the establishment of a bovine TB eradication program, the use of bovine purified protein derivatives (bPPD) for intradermal skin testing and *in vitro* testing, and the institution of federal regulations that govern the release of new bPPDs.

The United States Department of Agriculture's (USDA) bovine TB eradication program was started in 1917 in an effort to eliminate this zoonotic disease from livestock in the United States (Angus 1978). One major aspect of a good eradication program is the ability to correctly diagnose the disease under eradication. This requires the use of safe, reliable, and effective diagnostic tools. Federal regulations and guidelines have been instituted to ensure that bPPD is produced and tested according to a set of standard operating procedures.

Historically, standard operating procedures are developed from trial and error or adapted from previous work that has been conducted on the same subject. In the case of

bPPD, many of the guidelines involving product storage and handling appear to have been based off of experimental procedures involving the use of human PPD that is used to test humans for *Mycobacterium tuberculosis* infections.

The majority of published PPD stability studies have been specific for human PPD and conducted prior to the early 1980's. From the 1960s to the 1980s, two Canadian scientists working at Connaught Medical Research Laboratories, S. Landi and H. R. Held were the pioneers of human tuberculin PPD stability studies. Connaught Laboratories was the first manufacturer of Tubersol® (Connaught Tuberculin CT68), PPD made from *Mycobacterium tuberculosis* and used for human testing (vaccination news). Landi and Held conducted studies involving changes to the potency of Connaught Tuberculin when exposed to different preservatives, PPD precipitation agents, PPD temperature conditions, and PPD oxidation, just to name a few. The outcome of these studies led to recommendations that, for the most part, are still in place today involving human PPD and bPPD. One such recommendation by Landi and Held is that multi-use vials of human PPD, after initial use, be discarded after one month due to oxidation (Landi 1983). In fact, today's product insert for Tubersol® states the following: "A vial of TUBERSOL® which has been entered and in use for 30 days should be discarded because oxidation and degradation may have reduced the potency" (Tubersol® insert). On the other hand, the USDA guidelines involving bPPD state that 10.0 ml multi-use vials of bPPD must be discarded after two weeks after initial usage due to oxidation (VS Memo 552.11).

Up until recently, no studies have been conducted involving factors that may influence the potency of bPPD. In 2011, Maes *et al*. essentially revisited a study by Landi and Held involving the effect of different temperatures on the potency of PPD. In that particular study, human, bovine, and avian tuberculin PPD were all utilized in the evaluation. Results from this study indicated that bPPD did not experience a decrease in potency due to storage at 37 °C for 4 months and 100 °C for 1 hour (Maes 2011).

Little is still known concerning the factors that may influence the potency of bPPD. The aim of this study is to determine if bPPD, dispensed in multi-use vials, will retain potency after exposure to air for up to 6 months after initial usage when compared to bPPD multi-use vials that remained unopened. Like Maes *et al*, this research will revisit a prior study by Landi and Held, only this time will be specific to bPPD and will evaluate potential oxidative effects. Results from this study may lend support to further consideration relating to shelf life of opened multi-use bPPD bottles allowing for extended use in the field, less waste of product, and the need for additional vials due to the 2-week discard date. This study may also benefit *in vitro* diagnostics that utilize bPPD for bovine TB testing.

CHAPTER 2. LITERATURE REVIEW

Development of *Mycobacterium bovis* Purified Protein Derivative (bPPD)

In 1877, a veterinarian from Cornell University, James Law, advocated that bovine TB was transmissible to humans via inoculation or ingestion of undercooked meat or fresh milk that contained the TB bacilli (Miller 1989). Over ten years later in 1888, TB was recognized as being the major cause of death among humans, cattle and other animals in close contact with humans (Prichard 1988). Law received criticism from Robert Koch, a German bacteriologist, who made it his mission to find a cure for TB in humans because Koch felt that concentrating efforts into the eradication of bovine TB was wrong and that human TB was more important (Lochead 1928). In 1890, Koch announced that he had found a cure for TB in a product that he manufactured out of *Mycobacterium tuberculosis* cultures and glycerine, he called this product tuberculin (Gradmann 2006). Later that year, Koch stated that this cure or vaccine had no effect on patients that were healthy or suffering from another disease, and that TB infected individuals developed specific symptoms of fever and weakness with swelling and redness of infected tissue which diminished after 15 hours (Kaufmann 2000). Veterinarians in the United States (US) and other countries recognized these symptoms as a possible diagnostic tool and soon after his announcement; they began using Koch's tuberculin as a means to diagnose TB in cattle (Marshall 1932). In 1892, state and federal veterinarians in the US began using Koch's tuberculin for field testing cattle (Konya 1977).

Today, Koch's tuberculin has been replaced with a purified protein derivative tuberculin or PPD. The first PPD was developed by Florence B. Seibert in the US in 1934 (Siebert 1934). This PPD was made from a *Mycobacterium tuberculosis* culture precipitated with trichloracetic acid (Konyha 1977). Seven years later, Siebert and Glenn developed a new PPD from *Mycobacterium tuberculosis* cultures that was precipitated with ammonium sulfate in order to reduce nucleic acid content and yield a more pure PPD (Siebert 1941). During this time period, other countries were utilizing PPDs produced from *Mycobacterium tuberculosis*; however, the Netherlands in 1950 developed a bPPD precipitated with trichloracetic acid (Konyha 1977). In 1960, South Africa announced that all PPDs produced by the Onderstepoort Veterinary Institute, South Africa, would be made from *Mycobacterium bovis* strain AN-5 (Francis 1973). This decision was based off a cattle study in 1954, that compared *Mycobacterium bovis* PPD against PPD made from *Mycobacterium tuberculosis*; the result was that bPPD was more specific (Francis 1973). The United States, during this time period was still using a form of Koch's Old Tuberculin called Heat Concentrated Synthetic Medium (HCSM) tuberculin made by using a synthetic media instead of beef broth and heat-concentrating the *Mycobacterium tuberculosis* cultures (Francis 1973). Dr. A. Ranney, Chief, TB Eradication Section of the United States Department of Agriculture, commented that "if his country moves to the use of a PPD Tuberculin it will be of the bovine type" (Francis 1973). Finally, in the early 1970's, the US began using bPPD instead of HCSM (Konyha 1977). At the Veterinary Services Laboratories in Ames, Iowa, methods to produce ammonium sulfate derived bPPD from *Mycobacterium*

bovis AN5 were evaluated and the standard production methods were published in the Federal Register in early 1976 (National Archives).

Components of PPD

In 1941, Florence Siebert and John Glenn determined that the primary ingredient found in PPD was protein and to a lesser extent, carbohydrates (polysaccharides) and nucleic acids were also present (Siebert 1941). After testing different production methods, Siebert and Glenn developed a PPD composed of 92.9% protein, 1.2% nucleic acid, and 5.9% polysaccharides that they determined to be the standard for *Mycobacterium tuberculosis* PPD (Siebert 1941). Siebert concluded that the polysaccharide(s) may accompany some proteins through the precipitation step during production, and that they may serve only to protect the proteins because evidence supports that they are not responsible for causing a significant intradermal reaction (Siebert 1944). The nucleic acid found in PPD was isolated and by itself, was found to be non-antigenic (Siebert 1944). Siebert concluded that protein was the most important constituent of PPD because it elicited specific responses in humans and animals (Siebert 1944).

Angus, in 1978, described procedures for making reference bPPDs for veterinary use in the US. Ten lots of bPPD were produced with various levels of protein, nucleic acids, and carbohydrates. On average, nucleic acid and carbohydrate content for 1mg of protein was 1.76 µg and 0.45 µg respectively (Angus 1978).

In 1980, Landi and Held produced a batch of PPD called PPD-CT68 that was composed of 86.7% protein, 4.2% nucleic acid, 0.4% polysaccharides, and 1.4% lipids (Landi 1980). As of 2009, this master batch was still being utilized to produce Tubersol®, the PPD used to test humans for *Mycobacterium tuberculosis* (Tubersol® insert).

This information lends support that purified protein derivatives are mostly protein but not necessarily as pure, as the name implies.

The United States Department of Agriculture bovine TB eradication program

The US TB eradication program was started in 1917 (Angus 1978). Financial support for this program is provided by the federal government. A portion of the funding is used for slaughter surveillance, live animal testing, reagents, and indemnities. Surveillance and live animal testing is necessary for an eradication program and allows for states or zones to become classified based on the prevalence of bovine TB affected herds in their state or zone. A state or zone is classified into one of the five status categories: accredited – free, modified accredited advanced, modified accredited, accreditation preparatory status, and non-accredited (9 CFR Part 77). A state or zone's status is determined by the prevalence of TB affected herds in that state or zone. States or zones that have a higher risk of TB prevalence receive a lower status rating, therefore leading to more interstate moving restrictions placed on cattle originating from that state or zone. A state or zone's status is also an important aspect affecting the export, interstate movement, selling, or exhibition of cattle.

One major aspect of a productive eradication program is the ability to correctly diagnose and identify affected animals related to the specific program. According to the Uniform Method and Rules (UM&R 2005), the official tests for conducting bovine TB testing for cattle and bison is as follows and all require the use of a bovine PPD: caudal fold tuberculin test, comparative cervical tuberculin test, cervical tuberculin test, and bovine interferon gamma assay (cattle only) (UM&R 2005). Of these tests, the primary screening test is the caudal fold test.

Each fiscal year from 2006 to 2010, there were over one million caudal fold tests performed each year in the US with a slight decrease to under one million for fiscal year 2011 (personal communication with B. Hench). The product that is used to perform the caudal fold test is *Mycobacterium bovis* Purified Protein Derivative (bPPD). This product is supported by TB eradication program funds and distributed at no charge to accredited veterinarians and regulatory veterinarians performing program testing. The bPPD is typically distributed in bottles containing 10.0 mls with an expiration date of 30 months. Each multi-use bottle tests approximately 80 animals at best due to loss of product in the hub of single dose syringes. During the period from 2006 to 2011, over 100,000 bottles of bPPD were distributed from the National Veterinary Services Laboratories (NVSL) to test approximately 5 million animals (personal conversation with Brucella and Mycobacterium Reagents Team, NVSL) This estimate seems high, but in all sense, is probably low due to multiple testing locations and timelines, as well as guidelines placed on 10ml multi-use vials that requires disposal after 2 weeks of initial usage (VS memo 552.11).

Bovine PPD used to conduct testing in the US is obtained from a commercial supplier. Eligible suppliers must have their bPPD licensed by the Center for Veterinary Biologics (CVB) in order to be utilized in the US. There are specific guidelines set forth by the US Code of Federal Regulations that CVB follows to meet licensing requirements. Each commercial supplier must submit their specific outline of production to CVB prior to licensure consideration. Though the specific details of production are confidential, each supplier must meet the following specifications: (1) each bPPD must pass purity testing, (2) each bPPD must pass safety testing, (3) each bPPD must pass potency testing, (4) each bPPD must have a protein concentration of 0.90 mg/ml to 1.1 mg/ml, and (5) each bPPD must have a phenol concentration of 0.46 to 0.54 percent (9 CFR 113.26, 113.38, 113.409).

PPD Stability Studies

Once it was determined that Koch's tuberculin worked best as a diagnostic tool rather than a curative drug, individuals in many countries began work on improving the tuberculin process, leading to today's tuberculin Purified Protein Derivative (PPD). In order to ensure that PPD was produced in a safe and effective manner that would remain constant throughout each lot, stability and potency studies were conducted. For the first half of the 1900s, physicians, bacteriologists, and chemists were hard at work comparing Koch's Old Tuberculin with PPD. Media, buffers, preservatives, precipitation agents, time of culture growth, storage conditions, temperature, and light effects were studied because each of these conditions were thought to affect the outcome of the PPD quality, thus affecting the overall potency.

The World Health Organization (WHO) TB Research Office and the Biophysics Laboratory of the University of Copenhagen undertook an *in vivo* study involving the potency of human PPD after exposure to light. In March of 1952, 154 BCG vaccinated school children in Egypt were given the test PPD, which was exposed to light, in one arm and the control PPD, no exposure to light, in the other arm and comparisons were made based off of the diameter of induration in millimeters (mm) (WHO 1955). The test PPD was bottled into clear glass vials, containing 5 tuberculin units per 0.1 ml dose, and exposed to sunlight for 12 hours; the control PPD was not exposed to sunlight and made of the same concentration and the same quantity was given (WHO 1955). The results indicated that the sunlight exposed PPD produced smaller reaction sizes compared to the control PPD; therefore leading to the conclusion that light exposure caused the human PPD to become less potent (WHO 1955).

In the early 1960s, S. Landi and H. Held from the University of Toronto, Toronto Canada were very active in exploring all aspects of the composition, preparation, purification, and stability of human tuberculin PPD (Landi 1963, 1965, 1970, 1973, 1978,1981,1983). One of the earliest studies focused on two considerations involving tuberculin PPD stability. Landi studied the effects of various temperatures on the PPD and if lyophilization/freeze-drying would cause a change in stability (Landi 1963). Three dilutions of PPD were made and stored at 5 °C and 24 °C for 18 months with guinea pig potency testing occurring at 6, 9, 15, and 18 months (Landi 1963). To study the effects of freeze drying, the same three dilutions of PPD were made, bottled in 2ml aliquots, and frozen at -30 °C for 3 hours, lyophilized for 20 hours, and stored for 24 months at 5 °C, 24 °C, and 37 °C (Landi 1963). When compared

to the National Institutes of Health (NIH) reference tuberculin, there were no significant changes in potency for all experimental PPDs tested (Landi 1963).

Next, Landi and Held explored multiple precipitation methods for tuberculin PPD in order to find a product that was mainly protein with reduced nucleic acid content. After testing seven precipitation methods that used trichloracetic acid, ammonium sulfate, and chloroform, they found that trichloroacetic acid precipitation at 5 °C produced a higher yield of protein but, precipitation with ammonium sulfate produced a higher potency reaction in guinea pigs (Landi 1965).

To expand on the results found during the tuberculin precipitation study, Landi *et al*. looked at the different tuberculoprotein molecular weights found in PPD preparations to determine if there was an influence on potency. They found that in the different batches of tuberculin PPD studied, the molecular weights of the tuberculoprotein affected the potency of the PPD and that potency rather than molecular weight should be used to standardize tuberculin PPD preparations (Landi 1968).

Continuing their work in the early 1970s, Landi and Held evaluated PPDs that utilized chinosol as an anti-microbial preservative and those that utilized phenol. Their aim was to determine the stability of these preservatives in solution. The result was that phenol was superior due to a lesser degree of interaction with the components that make up the PPD, the lack of absorption to the rubber stoppers that seal the vials that contain the PPD, and the minimal evaporation rate through the rubber stoppers (Landi 1973).

From the early 1960s to the late 1970s, Landi and Held were the pioneers of human tuberculin PPD stability studies. The outcome of their findings paved the way for standardized PPD formulations. In 1978 they undertook a study to compare 15 commercially available PPDs prepared from a master batch of PPD and tested using 1, 5, and 250 tuberculin units under three different storage temperatures (Landi 1978). The results from this study showed that all dose strengths were stable for 3 years when stored at 4 °C, stable for 2 years when stored at 24 °C, and stable for 1 year when stored at 37 °C (Landi 1978). Their overall concluding remark based on their findings was "we agree with the Canadian and U.S. regulations that tuberculin products be stored at 2 °C to 8 °C (Landi 1978).

In the event that tuberculin PPD would succumb to storage conditions outside of the recommended 2 °C to 8 °C, Landi and Held showed that 24 °C to 34 °C conditions would cause no changes in potency for at least 1 year (Landi 1978). The next logical route for Landi and Held was to study the effect of extreme temperatures, -28 °C and 60 °C, on the potency of tuberculin PPD as well as if the phenol concentration would be affected. Their findings showed that at a dose of 5 tuberculin units per 0.1ml, stored at -28 °C for 91 days did not affect the potency or phenol concentration; however, potency and phenol was affected as early as day 5 for PPDs stored at 60 °C with a decrease in both (Landi 1981). Their recommendation was to continue to store tuberculin PPD at temperatures between 2 to 8 °C but, in the event that the PPD was inadvertently frozen, it still would be safe and effective to use if thawed completely (Landi 1981).

Tuberculin PPD is typically distributed in multi-dose vials. A needle attached to a syringe is used to withdrawal and administer a 0.1ml dose. During this process, air may be injected into the vial in order to make withdrawal easier. Also, during the bottling of the tuberculin PPD final product, the PPD may not completely fill the vial, therefore allowing air to remain in the headspace of the vial. In 1983, Landi and Held wanted to determine if air had an effect on the potency of a 5 tuberculin unit dose of PPD and if air had an effect on lyophilized PPD (Landi 1983). Their findings indicated a loss of approximately 50% potency over the course of 4 months when stored at 5 °C for those vials containing air in the headspace (Landi 1983). For the vials that were lyophilized, sealed with cotton plugs to allow free access to air, and stored at 24 °C for seven years, the lyophilized PPD could not be reconstituted and therefore unusable (Landi 1983). The outcome of this work was the following recommendations: when manufacturing PPD, the vials must be filled completely, that multi-dose vials may lose potency, and that any vial stored longer than one month with a headspace larger than the fill space be discarded (Landi 1983).

Over 25 years later, in 2011, Maes *et al*. revisited the effect of temperature on tuberculin PPD. One aspect of this study focused on the stability of bPPD at 37°C and 100°C. Their study was similar to the ones conducted by Landi and Held in 1978 and 1981. In this study, the bPPD was exposed to 37°C for 3 months and 100°C for 1 hour and potency testing was conducted in infected cattle (Maes 2011). They concluded that bPPD is heat stable, without a loss of potency. They credited this outcome to a step in the bPPD production process that calls for heating of the initial cultures prior to precipitation

therefore producing a product that is composed of denatured, thermo-stable proteins (Maes 2011).

Concerns involving the use of proteins for pharmaceuticals

A drug is defined as "a product used in the diagnosis, cure, treatment, or prevention of a disease" (Merriam-Webster 2002). According to this definition, purified protein derivatives (PPDs) would be considered a drug. Pharmaceutical companies study the stability of drug products in great length in order to release a new drug that is safe and effective for the consumer. Many new drug products are being discovered that are proteins or that specifically target proteins, therefore, it is important to understand the stability of protein drugs, like PPDs, and what may affect their shelf life.

Protein stability may be affected by proteolysis, deamidation, and oxidation, which are categorized as chemical instabilities; whereas, aggregation, precipitation, denaturation, and adsorption to surfaces are considered physical instabilities (Manning 1989). Proteolysis involves the breakdown of proteins into smaller peptides through hydrolysis of the peptide bonds by enzymes (Berg, et al). Deamidation is considered the most common chemical degradation pathway of proteins and peptides and involves the hydrolysis of asparagine and glutamine side chain amides and may be controlled by adjusting pH conditions (Manning 2010). If proteins unfold due to deamidation, amino acid groups that normally would not be exposed to outside contacts, now are exposed, which may lead to an increase in immunogenicity (Cleland 1993). Proteins that contain the following amino acids are subject

to oxidation: histidine, methionine, cysteine, tyrosine, and tryptophan (Manning 2010). Protein oxidation occurs either directly by reactive oxygen species or indirectly by reaction with secondary by-products of oxidative stress and can affect all of the amino acyl side chains through different types of reactive oxygen species like superoxide anions, hydrogen peroxide and hydroxyl radicals (Shacter 2000). Atmospheric oxygen has been found to oxidize methionine (Manning 1989). Oxidation of protein drug products can occur during production, purification, formulation, and storage (Manning 2010). The outcome of protein oxidation may be an increase in aggregate formation (Manning 2010) and proteolysis therefore leading to altered immunogenicity (Shacter 2000). Aggregation of protein pharmaceuticals can be challenging to overcome because it can occur during refolding, purification, sterilization, shipping, and storage of protein products even under favorable neutral pH and 37°C temperature conditions (Chi 2003). Proteins may have limited stability in solution and because of this, become denatured leading to aggregation (Cleland 1993). Aggregates can cause adverse immunogenic effects, reduced efficacy, and the protein solution can appear turbid or physically separate from solution (Manning 2010). Precipitation or particle formation within protein suspensions may result in the most immunogenic particles found in these suspensions; this is not only due to aggregation but also due to the addition of a solute that affects the chemical composition of the protein to exceed the solid phase, which is fully reversible after dilution (Manning 2010). Denaturation can occur with temperature or pH changes and will cause the protein's tertiary structure to unfold, leading to changes in physical and biological properties (McMurry 1999). The loss of structure is a physical change but, the composition of the

protein remains the same (Manning 2010). The most common protein denaturation process is caused by heat and most often is irreversible due to the formation of aggregates (Manning 2010). In this instance, aggregates are formed from the association of unfolded proteins that have lost their globular structure due to high temperature (Manning 2010).

Adsorption, another physical instability, occurs when proteins adhere to hydrophobic surfaces; this becomes important when choosing bottling equipment and containers to store and package protein products to avoid loss of proteins within a solution (Cleland 1993). Many of these chemical and physical instabilities are caused by factors that may or may not be manageable according to pharmaceutical design and method of action, therefore leading to very specific handling and use instructions.

Protein stability of aqueous solutions used for drug therapies can be affected by temperature, pH, salts, and preservatives, just to name a few (Chi 2003). All of these factors have to be considered when manufacturing protein products. The thermodynamic stability of native protein conformations is weak and therefore temperature fluctuations can affect the protein's native structure (Chi 2003). Cleland reported that a protein may undergo deamidation and the formation of aggregates at temperatures above 39 °C or below 30 °C, depending on the protein (Cleland 1993). The pH of a protein solution determines if the protein has a negative or positive charge; aggregation may be prevented if protein solutions maintain a narrow pH range (Chi 2003). A way to reduce factors that contribute to chemical degradation is to adjust the protein solution pH to between 5 and 7 (Cleland 1993). Choosing a pH solution that will limit redox reactions will limit the ability for

oxidation to occur (Shacter 2000). Buffers containing certain salts like phosphate may increase the stability of proteins by their ability to directly bind to the protein (Manning 2010). The binding of salts to proteins may also be affected by pH conditions (Chi 2003). Glycerol and sugars are known to stabilize proteins at low concentrations because water molecules pack around the protein; however, stability decreases when the amount of glycerol and sugar increases (Manning 1989). Preservatives are used in some pharmaceuticals to prevent contamination by microbes. Phenol and benzyl alcohol are common preservatives added to protein solutions to ensure sterility of a multi-use product, unfortunately, depending on the product, each one of these preservatives may induce protein aggregation (Chi 2003).

Proteins in solutions are being used to diagnose and treat certain diseases. However, due to the nature of proteins, they may become unstable in solution. It is extremely important to know all the factors that may have an influence on proteins to undergo physical and chemical changes leading to products that are unstable, unsafe, and unreliable.

Diagnostic Assays that Utilize bPPD

Delayed-type hypersensitivity tests

Delayed–type hypersensitivity is a cell-mediated immune response that occurs in the dermis as a result of a particular antigen being intradermally injected. In this particular situation, the antigen is bPPD. Injection of bPPD initiates inflammation leading to the delayed-type hypersensitivity response in TB infected animals. This “delayed” response

occurs within 24-72 hours after antigen exposure. However, γδ T cells are present as early as 6 hours post-injection, at the site of injection (Doherty 1996). Leucocyte adhesion molecules become expressed leading to an influx of antigen-specific lymphocytes, neutrophils, and macrophages, as well as an accumulation of fibrin deposits to the site of injection, therefore, contributing to the inflammation of localized blood vessels, known as leucocytoclastic vasculitis (Doherty 1996). An increase in the presence of lymphocytes and neutrophils occur between 12 – 24 hours and around 72 hours, inflammatory edema is evident (Doherty 1996). Antigen specific lymphocytes - T_H1 CD4 cells receive antigen from macrophages in the form of peptide MHC class II presentation, and in turn, release inflammatory cytokines (IFN-γ and TNF-β) (Janeway). These cytokines cause an increase in the permeability of local blood vessels allowing an increase in fluid, as well as additional inflammatory cells and phagocytes to migrate to the injection site causing a visible localized skin response (Janeway). The classic delayed-type hypersensitivity reaction is the tuberculin skin test.

There are three types of delayed – type hypersensitivity tests that are approved for official use by the United States TB eradication program: the caudal fold test (CFT), the comparative cervical test (CCT), and the single cervical test (CT) (UM&R 2005). The caudal fold test is used for routine screening of cattle and bison (UM&R 2005). A 0.1ml dose of bPPD at 1.0 mg/ml is injected intradermally in the caudal fold area and the reaction, if any, is measured 72 hours later. Any reaction categorizes an animal as a suspect and follow up confirmatory testing, usually the CCT, is performed (VS Memo 552.15). A reaction may

mean that the animal is infected with *Mycobacterium bovis* or that the animal has been exposed to other closely related environmental Mycobacteria or related genera of bacteria.

The CCT is another intradermal test that is administered in the cervical region. It is used to determine if the CFT was truly positive for *Mycobacterium bovis* or a reaction to *Mycobacterium avium*. A 0.1ml dose of bPPD at 1.0 mg/ml and a 0.1ml dose of *Mycobacterium avium* PPD (aPPD) at 0.4 mg/ml is injected in separate locations on one side of the cervical region and changes in skin thickness is measured 72 hours later (UM&R 2005). Avian measurements are subtracted from bovis measurements and the result is plotted on a CCT scattergram specifically developed for cattle, bison, and cervidae (VS Memo 552.15). CCT suspects must be retested no less than 60 days from the initial CCT or sent for slaughter and animals that are classified as CCT suspects twice, consecutively, are determined reactors (VS Memo 552.15). The period of 60 days or less is considered a transitory period when the delayed-type hypersensitivity response may become dampened due to the desensitization of effector T_H1 lymphocytes to the bPPD therefore leading to the possibility of inaccurate skin test results (Doherty 1995). Therefore, it is necessary to re-test after 60 days.

The single cervical test uses a double strength bPPD at 2.0 mg/ml to test known exposed cattle and bison. A 0.1ml dose is intradermally injected into the cervical region and changes in skin thickness are measured 72 hours later. Any response classifies that animal as a reactor (VS Memo 552.15).

Gamma interferon assay

The gamma interferon (IFN-γ) assay is used as an official supplemental diagnostic test in the US bovine TB eradication program (UM&R 2005). The commercially available Bovigam® is an *in vitro* enzyme-linked immunosorbent assay (ELISA) used for detection of IFN-γ produced by lymphocytes in response to separate stimulations of bPPD and aPPD. The assay requires that whole blood be collected from cattle within 3 – 30 days after intradermal skin testing. The blood collected from one animal is placed into 4 separate wells of a 24-well plate. The blood from one well is stimulated with bPPD, the second well is stimulated with aPPD, the third well is stimulated with pokeweed mitogen. A nil (no antigen) is added to the fourth well. Stimulations occur within 30 hours of collection. The plate is incubated at 39 °C for 16 – 24 hours, centrifuged, and the plasma is harvested from each well. The pokeweed mitogen is used to determine the viability of the whole blood lymphocytes to ensure that they are able to produce IFN-γ upon stimulation with the PPDs. The nil is usually a buffer that is used as a non-antigenic negative control. The nil should not cause any lymphocytes to produce IFN-γ.

At this point, the plasma may be frozen for testing at a later date or used the same day of collection (Bovigam® product insert). The next step in the testing process is to add the plasma to the pre-made kit ELISA plates. Each well of the kit plate contains bound anti-bovine IFN- γ monoclonal antibodies. The bPPD stimulated plasma is added to one well, aPPD stimulated plasma is added to a second well, pokeweed mitogen stimulated plasma is added to a third well and nil plasma is added to a fourth well. After the plasma is added,

another anti-bovine IFN-γ monoclonal antibody containing an enzyme substrate (used for color detection) is added. Any IFN-γ contained in the plasma samples will bind to the anti-bovine IFN-γ monoclonal antibodies bound on the ELISA plate. The enzyme labeled anti-bovine IFN-γ monoclonal antibodies will also bind to any IFN-γ that is adhered to the ELISA plate. The ELISA is performed at an optical density (OD) of 450 nm. The degree of color production is correlated to the amount of bound IFN-γ. OD values are generated from each well (Bovigam® product insert). A positive result indicates that the cattle being tested are infected with *Mycobacterium bovis*. In order to determine if the sample is positive, two criteria must be met: (1) the OD value of the "stimulated" nil plasma is subtracted from the OD value of the stimulated bPPD plasma and the value must be greater than or equal to 0.1 and (2) the OD value of the stimulated aPPD plasma is subtracted from the OD value of the stimulated bPPD plasma and the value must be greater than or equal to 0.1; if both criteria are met, the sample is positive (Bovigam® product insert). However, for the US TB eradication program, classification of these test animals (suspect or reactor) is based on the UM&R and under the discretion of Designated TB epidemiologists or Regional TB epidemiologists.

Testing standards for new bPPDs

In order for bPPD to be used as a diagnostic tool for the USDA bovine TB eradication program, it must pass a purity test, a safety test, a potency test, a protein test, and a phenol test. Regulations governing all of these tests are set forth in the United States Code of Federal Regulations Part 9 (9 CFR) 113.26, 113.38, and 113.409. The following sections are

a direct reference to the federal regulations because there are no other federally approved methods to release a new lot of bPPD.

Purity Testing 9 CFR 113.26

A new lot of bPPD that is manufactured must be tested to ensure that it is free of all viable bacteria and fungus. The 9 CFR guideline states that fluid thioglycollate medium with or without 0.5 percent beef extract shall be used to test for viable bacteria and soybean-casein digest medium shall be used to test for fungus. Ten samples of the new bPPD will be used to inoculate 10 containers of fluid thioglycollate medium and 10 containers of soybean-casein digest medium. Fluid thioglycollate medium test samples are incubated at 30 °C to 35 °C for 14 days and soybean-casein digest medium test samples are incubated at 20 °C to 25 °C for 14 days. If any growth is present, one re-test is allowed and if that test is found to support growth of bacteria or fungus, the new bPPD in ineligible for release to conduct bovine TB testing.

Safety Testing 9 CFR 113.38

Two guinea pigs each receive a 2.0 ml injection of a new lot of bPPD either subcutaneously or intramuscularly and observed for 7 days unless otherwise specified by the bPPD manufacturer's outline of production. If either guinea pig experiences any adverse reactions that are associated with the bPPD, the new lot is considered unsatisfactory and may not be released to conduct bovine TB testing.

Potency Testing 9 CFR 113.409

A test bPPD is tested against an approved reference bPPD, a total of 43 white female guinea pigs that weigh 500 – 700 grams are used per test, 20 guinea pigs are inoculated with *Mycobacterium bovis* (*M. bovis*) sensitinogen where half will be used to test the reference bPPD and the other half will be used to test the new bPPD, 20 guinea pigs are inoculated with *Mycobacterium avium* (*M. avium*) sensitinogen where half will be used to test the reference bPPD and the other half will be used to test the new bPPD and the remaining 3 guinea pigs are not sensitized and used as controls for the test bPPD. Thirty-five days after injection of sensitinogen, dilutions of 0.6, 1.2, 2.4, and 4.8 micrograms are made of the bPPD and the reference bPPD and each one is randomly injected intradermally into their assigned guinea pig. Measurements are made 24 hours post-injection and each area of erythema is recorded. All four dilution site measurements per guinea pig are added together to obtain one final measurement for each animal, and then the 10 final measurements for each bPPD sensitized group are added together, divided by 10, and an average measurement response is obtained. A total of 4 final measurements are generated. From these measurements, a specificity index is determined. The average measurement from the test aPPD group is subtracted from the average measurement of the test bPPD, the value obtained is the specificity index for the test bPPD. The specificity index for the reference bPPD is obtained in the same manner. For the potency test to be considered valid the specificity index for the reference bPPD must by a value of 400 mm^2 or greater and if it is less, the test is considered not valid and must be repeated with a new

group of guinea pigs. If the test is valid, the specificity index of the test bPPD must be at least 440 mm^2 to receive a satisfactory rating. If the specificity index is 360 mm^2 or less, the test is unsatisfactory and the new lot of bPPD cannot be used for bovine TB testing. If the specificity index falls between 360 mm^2 and 440 mm^2 the test is considered inconclusive and may be repeated with a new group of guinea pigs or deemed unsatisfactory. The test bPPD is also considered unsatisfactory if any of the 3 unsensitized control guinea pigs develop erythema reactions.

Protein Testing 9 CFR 113.409(d1)

Final products of bPPD must contain a protein concentration of 0.90 mg/ml to 1.1 mg/ml. Protein concentration is determined by the Micro-Kjeldahl Test.

Phenol Testing 9 CFR 113.409(d2)

The percentage of phenol in each new lot of bPPD must be within the range of 0.46 to 0.54 percent. Phenol concentration is determined by titration with a standardized bromide-bromate solution.

CHAPTER 3. MATERIALS AND METHODS

Bovine Purified Protein Derivatives (bPPDs)

United States Department of Agriculture (USDA) bPPD lot 10049X with a protein concentration of 1.08 mg/ml was used. This lot was tested for potency and sterility by the USDA Center for Veterinary Biologics (CVB) and released for use by the USDA for bovine TB testing. Multi-use vials were exposed to air via direct injection of 1.0 ml of air into each vial and subsequent withdrawal and discard of 5.0 mls of bPPD, leaving an air filled head space containing half air and half bPPD. Multi-use vials of bPPD treatment groups were produced in duplicate, aged for a specific time under 4°C, and comprised the following: bPPD positive control (6 months air exposure), bPPD A (6 months air exposure), bPPD B (4 months air exposure), bPPD C (3 months air exposure), bPPD D (2 months air exposure), bPPD E (1 month air exposure), bPPD F (2 weeks air exposure), and bPPD negative control reference (no air exposure). The bPPD positive was established by creating an extreme oxygen environment within the PPD, more so than the other treatment bPPDs, and more so than under normal testing conditions in the field. Sterile filtered air was pumped into each bottle for no less than one hour within a Biological Safety Cabinet (BSC). The air hose was attached to a sterile 0.22µm filter that was attached to an 18g x 1 inch needle inserted through the rubber seal directly into the bottle containing the bPPD. A smaller 22g x 1 inch needle was also placed into the rubber seal to allow air to be released. The bPPD positive

were established to create a positive control. Unopened vials of bPPD that were never exposed to air were used as negative controls or references.

Guinea Pigs

A total of 24 white-haired, non-pregnant guinea pigs ranging in weight from 500 to 700 grams were used. Twenty guinea pigs were inoculated with 0.25 mls of *Mycobacterium bovis* sensitinogen lot 31-S 0601 prepared by the Brucella and Mycobacterium Reagent Team, National Veterinary Services Laboratories, Ames Iowa. Inoculations took place intramuscularly (IM) in both hind legs using a 3 ml syringe with a 20 gauge by 1 inch needle. Four guinea pigs were used as controls and not inoculated with sensitinogen. On day 35, each guinea pig was bathed and hair was removed from their abdomens to the inside of each leg.

For this potency test, a modified version of the 9 CFR was used. The first modification was to not sensitize any guinea pigs with *Mycobacterium avium* sensitinogen due to prior 9 CFR testing which indicated that this lot met the specificity index requirements. The second modification was to omit using a known bPPD reference. Again, due to prior testing which utilized a known bPPD reference, the unopened negative control vials were used as the reference in this test. The third modification was to inject all treatment bPPDs into each guinea pig at the same dilution. By evaluating potency in this manner, each individual guinea pig's level of response to each treatment bPPD would be specific to that guinea pig.

All 8 bPPDs were injected intradermally on the abdomen of each guinea pig starting at the posterior end using groups of two bPPDs and working towards the anterior end of the abdomen. Top row posterior end to anterior end: bPPD pos, bPPD A, bPPDB, and bPPD C. Bottom row posterior end to anterior end: bPPD neg, bPPD D, bPPD E, and bPPD F. The bPPDs were not randomized. Guinea pigs were divided into 4 groups containing 6 guinea pigs each at the time of bPPD challenge. Group 1 received each bPPD at a dilution of 4.8µg/0.1ml. Group 2 received 2.4µg/0.1ml. Group 3 received 1.2µg/0.1ml and Group 4 received 0.6µg/0.1ml. Each bPPD was injected using a 1 ml syringe containing a 26 gauge by 3/8 inch needle. On day 36, exactly 24 hours after challenge, reaction site measurements were taken to the nearest tenth millimeter (mm) vertically and horizontally using an automated caliper. Horizontal and vertical measurements were multiplied together to achieve a final measurement in mm^2.

Cattle

Seven male Holstein steers approximately 1 year old were inoculated via aersolization with 10^4 cfu of *Mycobacterium bovis* strain 95-1315 (Michigan WTD isolate) within a BSL -3 animal facility. The inoculation procedure has been previously described by Palmer *et al* (Palmer 2002). The cattle were infected 90 days prior to bPPD injections. For this potency test, all treatment bPPDs were injected into the cervical region, 4 bPPDs on the right side and 4 bPPDs on the left side. No avium PPD was used. Prior to injection, a patch of hair was clipped from each injection location. Pre-injection site measurements were taken to the nearest tenth mm from each test location on the cervical region using a caliper

prior to injection of the treatment bPPDs. Injections of bPPDs were randomized with injections occurring at the following cervical locations on each Holstein: Right Dorsal Anterior, Right Ventral Anterior, Right Dorsal Posterior, Right Ventral Posterior, Left Dorsal Anterior, Left Ventral Anterior, Left Dorsal Posterior, and Left Ventral Posterior. Each Holstein received a dose of 0.1 mg/0.1ml of each bPPD. A 1.0ml syringe with a 26 gauge by 3/8 inch needle was used for each injection. Reaction site measurements to the nearest tenth mm were obtained 72 hours post injection using a caliper and subtracting the pre-measurement readings to obtain a final measurement in mm.

Gamma Interferon Production

The commercial Bovigam® assay was used to determine gamma interferon production. Blood was obtained from each of the 7 Holsteins described previously prior to injection of the 8 experimental bPPDs. Blood was also obtained from 2 cows that were not sensitized or infected with *Mycobacterium bovis*. 1.5 mls of blood from each animal was placed in 4 separate wells of a 24-well plate for stimulation. For each animal, 4 wells containing blood were needed for the assay. One well was stimulated with bPPD, one well was stimulated with avium PPD, one well was stimulated with Pokeweed mitogen and one well was stimulated with nil (contains no antigen). A 100 µl sample of each experimental bPPD, avium PPD, Pokeweed mitogen, and nil was used to stimulate the blood in duplicate. Plates were incubated at 39°C for 17-22 hours. After incubation, each plate was centrifuged at 2000 rpm for 15 minutes and plasma was removed. Plasma was stored at -70°C for 55 days. All bPPDs, avium PPDs, and Pokeweed mitogen were used at a

concentration of 15.0 µg/0.1ml. The Nil was phosphate buffer saline. When the ELISA was performed, plasma was allowed to thaw, vortexed briefly to mix, and then added to the pre-made Bovigam® kit ELISA plates. The assay was performed according to the manufacturer's instructions.

Protein Analysis

A sample of each experimental bPPD and a newly opened bPPD negative control reference was sent to the Chemistry and Analytical Section of the National Veterinary Services Laboratories for protein determination. The protein concentration of each experimental bPPD was compared to the bPPD negative control reference. Each bPPD was tested in triplicate. The Micro Kjehdahl method is the approved test for determining the protein concentration of manufactured PPDs according to the United States Code of Federal Regulations Title 9 CFR 113.409(9CFR 113.409).

pH

Each experimental bPPD and a newly opened bPPD negative control reference was pH tested and results were compared to the bPPD negative control reference. A calibrated Thermo Electron Orion 3 Star pH Benchtop Meter was used. Values were recorded using two significant digits.

Visual Inspection

Each experimental bPPD was visually compared to the bPPD negative control reference bPPD and inspected for color changes and precipitate formation.

Statistical Analysis

All statistical analyses were performed using a one-way analysis of variance (ANOVA) with a .05 significance level. Dunnett's Multiple Comparison Test was also utilized to compare all bPPD treatment groups with the bPPD negative control reference. The mean values for each treatment bPPD were analyzed.

CHAPTER 4. RESULTS

Guinea Pig Intradermal Skin Test

All 20 guinea pigs that were sensitized developed red swollen reaction diameters at each site of injection for each dilution (Figure 4.1). All control guinea pigs that were not sensitized, showed no reactions. Responses were measured by using an automated caliper, with readings to the tenth decimal place and calculated in accordance with 9 CFR. All

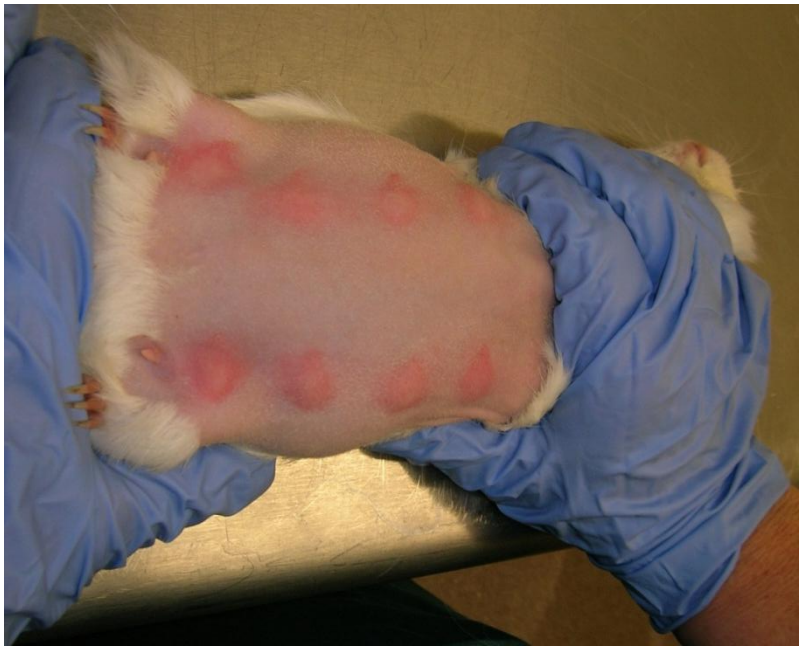

Figure 4.1. The abdomen of a guinea pig with positive delayed-type hypersensitivity reactions to all bPPDs. Top row left to right: bPPD pos (6 months), bPPD A (6 months), bPPD B (4 months), and bPPD C (3 months). Bottom row left to right: bPPD negative reference (0 months), bPPD D (2 months), bPPD E (1 month), and bPPD F (2 weeks).

dilution set measurements for each specific treatment bPPD were added together and a final mean response in mm^2 was obtained per treatment bPPD (Figure 4.2).

The one-way ANOVA indicated that there was a significant difference between at least 2 of the bPPDs due to a P value < 0.05. However, when further analysis was conducted using Dunnett's Multiple Comparison Test, comparing each of the treatment bPPDs to the bPPD negative control reference, there was no significant difference at a 95 percent confidence level. The lowest mean response of 122.1 mm^2 was from bPPD D, which had been exposed to air for 2 months. The largest mean responder was 169.0 mm^2 from bPPD C, which had been exposed to air for 3 months. The remaining treatment bPPD responses were: bPPD positive = 143.6 mm^2, bPPD A = 168.0 mm^2, bPPD B = 165.7 mm^2, bPPD E = 126.3 mm^2 and bPPD F = 129.4 mm^2, and bPPD negative reference = 131.4 mm^2.

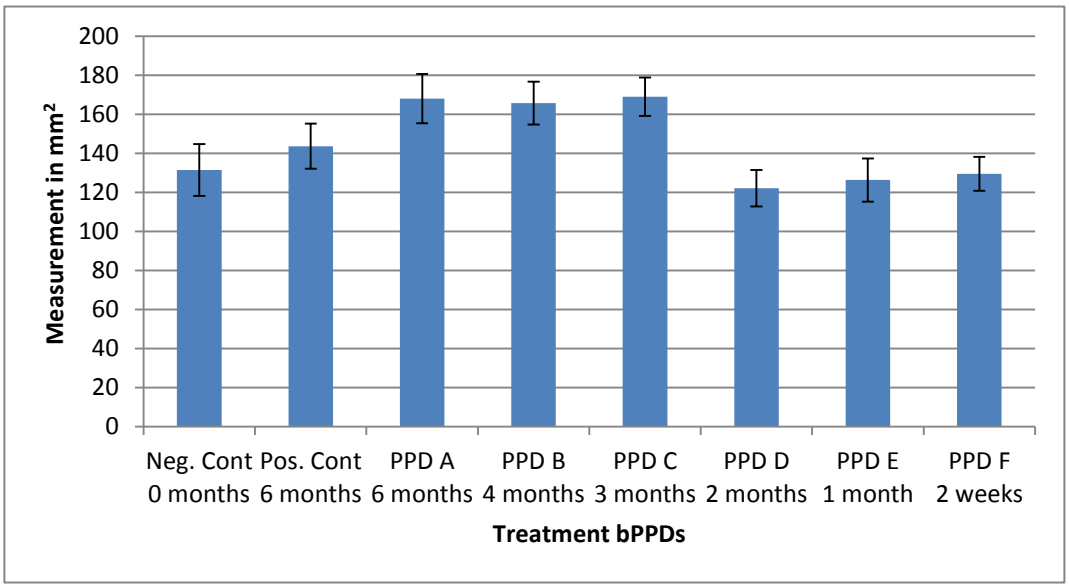

Figure 4.2. Mean (SEM) guinea pig responses in mm^2. Sensitized *Mycobacterium bovis* guinea pig responses to each treatment bPPD (n=20). Measurements were taken 24 hours after injection of bPPDs.

Cattle Intradermal Skin Test

All seven cattle developed swollen, palpable reaction responses (Figure 4.3). Each treatment bPPD response from each animal was added together and a final mean response was obtained in mm for each treatment bPPD (Figure 4.4). There was no significant difference between all bPPDs with a p value > 0.05. The lowest mean response of 21.2 mm was from bPPD D, which had been exposed to air for 2 months. The largest mean responder was 26.0 mm from PPD negative, which had never been exposed to air. The remaining treatment bPPD responses were: bPPD positive = 21.4 mm, bPPD A = 21.9 mm, bPPD B = 24.1 mm, bPPD C = 25.0 mm, bPPD E = 22.7 mm, and bPPD F = 22.4 mm.

Figure 4.3. Cattle intradermal delayed-type hypersensitivity reactions in response to bPPD injections. The cervical region of a cow with 4 palpable reaction sites. Four treatment bPPDs were randomly injected on one side of the cervical region and 4 treatment bPPDs were randomly injected on the opposite side.

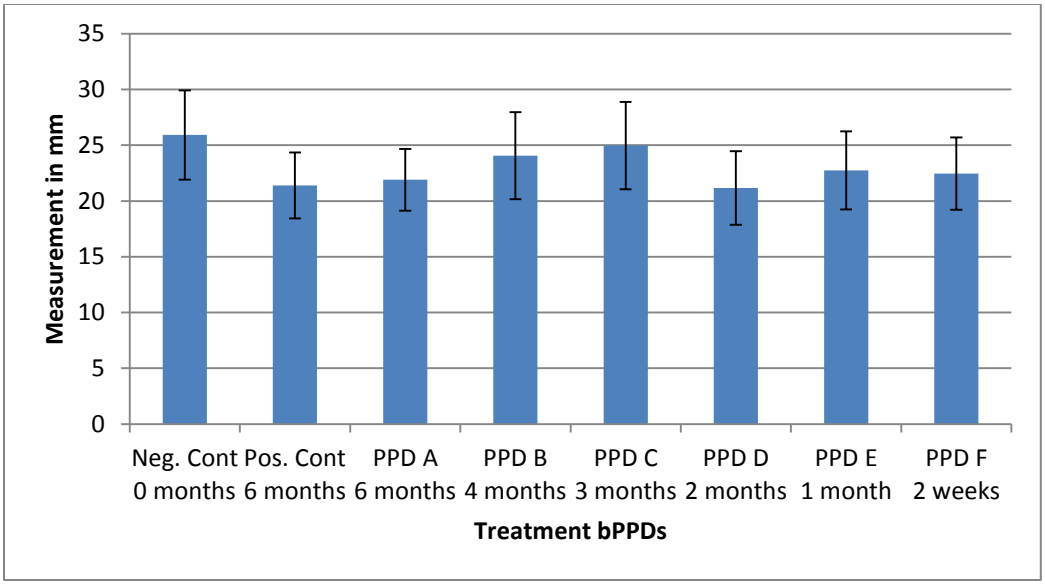

Figure 4.4. Mean (SEM) cattle responses. *Mycobacterium bovis* infected cattle responses to each bPPD in mm (n=7). Results were obtained from measuring the reaction site and subtracting the value of the initial skin thickness reading prior to injection of bPPDs.

Gamma Interferon Production

All treatment bPPDs were able to effectively stimulate blood samples that were collected from the 7 experimentally infected cattle to produce IFN-γ. Each animal's blood sample was treated with each treatment bPPD in duplicate and optical density (OD) readings at 450 nm were generated from the plasma. The two OD readings from each animal were added together and a final mean OD value was obtained for each treatment bPPD (Figure 4.5). There was no significant difference between all bPPDs at a p value > 0.05. The lowest mean OD value 1.09 was from bPPD F, which had been exposed to air for 2 weeks. The largest mean OD value was 1.31 from PPD B, which had been exposed to air for 4 months. The remaining treatment bPPD responses were: bPPD negative reference = 1.23, bPPD positive = 1.24, bPPD A = 1.27, bPPD C = 1.14, bPPD D = 1.20 and bPPD E = 1.16.

All bPPDs were unable to stimulate blood lymphocytes to produce IFN-γ collected from 2 cows that were not sensitized with *Mycobacterium bovis* sensitinogen or experimentally infected.

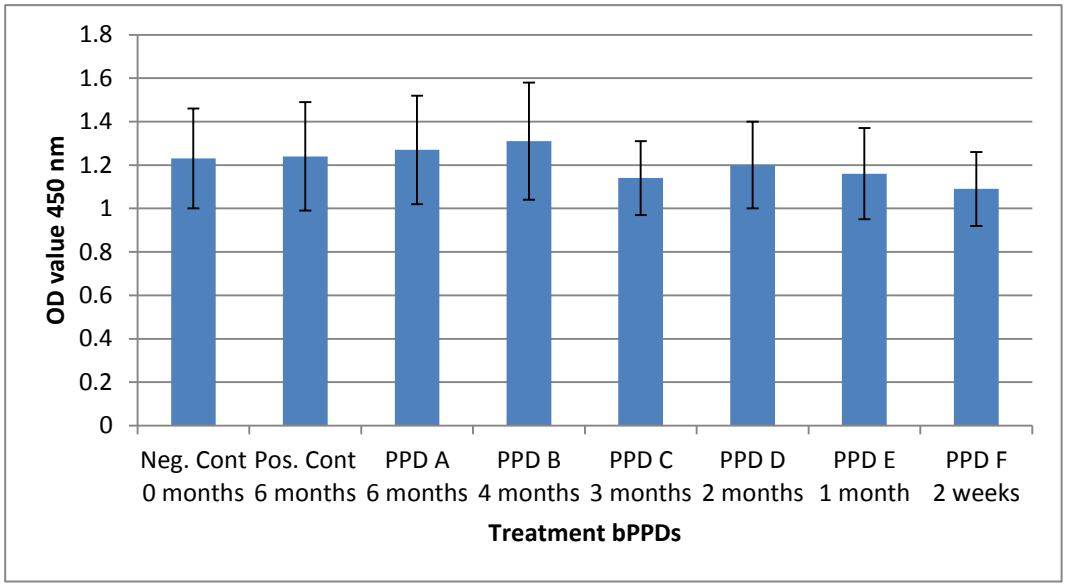

Figure 4.5. Mean (SEM) OD values for in vitro production of IFN-γ. Blood lymphocytes from *Mycobacterium bovis* infected cattle were stimulated with each treatment bPPD (n=7).

Protein Analysis

Prior to initial release of the bPPD lot 10049X, the protein concentration was 1.08 mg/ml. After treatments, each bPPD was tested in triplicate with an average result of 1.10 mg/ml for bPPDs: Negative control reference, Positive, bPPD B, bPPD C, bPPD D, bPPD E, and bPPD F. The bPPD A had an average result of 1.00 mg/ml. These values remained consistent after exposure to air with no loss in protein concentration and remain within the acceptable 0.90 mg/ml to 1.1 mg/ml range according to the 9 CFR.

pH and visual inspection

Each bPPD was tested at 24.1 °C +/- 0.1 °C. The pH values for each PPD were 7.32-7.33 compared to the PPD negative control reference value of 7.33. These values indicated that there were no changes to the treatment bPPDs pH when exposed to air.

Each treatment bPPD, including bPPD negative reference contained no precipitates that had fallen out of solution, there were no signs of turbidity, and there was no variation of color.

CHAPTER 5. DISCUSSION

The results of this study indicate that multi-use vials of bovine tuberculin PPD (bPPD) retain a consistent potency level after initial use for up to 6 months following air exposure. Treatment bPPDs were potency tested *in vivo* in guinea pigs and cattle to measure the cell-mediated delayed-type hypersensitivity responses and *in vitro* by the commercial Bovigam® kit to measure gamma interferon production by blood lymphocytes stimulated with bPPDs. Protein concentration, pH, as well as visual inspection of the treatment bPPDs were also important considerations for this study. All three potency tests, as well as protein determination, pH, and visual inspection, produced results that were consistent in concluding that bPPDs retained acceptable potency for up to 6 months after being exposed to air, stored at 4°C, and in the original amber colored multi-use vials.

Unlike other studies that involve the use of a known positive control to measure test validity, there was no such information available to create a known positive control for this study. The oxidation study by Landi and Held indicated that PPD had a 50 percent reduction in potency after 4 months of air exposure (Landi and Held 1983). Therefore, it was hypothesized that a decrease in potency would be observed by the extreme treatment of the bPPD positive with air, thus creating a feasible bPPD positive control for this study. However, this proved not to be the case because there was no significant difference between the proposed bPPD positive and the bPPD negative.

A p value < 0.05 for the guinea pig potency assay indicated that at least 2 bPPDs were significantly different. When all bPPDs were compared solely to the bPPD negative control reference, there was no significant difference and the p value was > 0.05 for each treatment bPPD. Review of the measurement data indicated that the bPPDs that had been exposed to air the longest were larger, but still insignificant, than the bPPD negative control reference. It is unclear why this was the case. In regards to the locations of each bPPD (Figure 4.1), the sites were not randomized. The largest responders: bPPD positive, bPPD A, bPPD B, and bPPD C were administered to the top row of every guinea pig. Without considering the specific bPPD samples injected in this area, it would appear this area of the guinea pig was more reactive. However, this is most likely not the case. The epidermis corresponding to the top row injection area is anatomically similar as the bottom row injection area and any differences contributing to increased measurements would be negligible.

Another consideration would be oxidation due to exposure to air. However, to further investigate, the proteins that compose the final product of bPPD would need to be known. Unfortunately, there is still very little data available specifying all the proteins that compose the final product of bPPD. However, one known protein is MPB70 (Rennie 2010). This protein contains the amino acids histidine, methionine, cysteine, and tyrosine (Wiker 1988), all of which are prone to oxidation (Manning 2010). Since protein oxidation may lead to altered immunogenicity due to an increase in aggregation and proteolysis (Shacter 2000), could the bPPD responses be contributed to oxidation? If this were the case, it would

also be expected that these reactions would be seen in the other *in vivo* test that utilized cattle. However, this was also not the case; there were no significant differences between all treatment bPPDs in the cattle study.

Based on the combination of all data presented in this study, and the lack of additional information pertaining to known proteins that compose the final bPPD, the larger guinea pig responses generated by the longest air exposed bPPDs can most likely be contributed to lack of randomization, and not a result of oxidation or anatomical location.

These results differ from Landi and Held who determined in 1983 that exposure of human PPD to air caused a loss of potency in guinea pigs due to oxidation and that the product must be disposed of after 1 month (Landi 1983). To this author's knowledge there have been only two studies conducted involving the effects of oxidation on tuberculin PPD; the study by Landi and Held and this current study. There were differences in the experimental setup of each study but not enough data was available from the Landi and Held study to draw specific conclusions comparing and contrasting the studies to pinpoint what contributed to the differences. One observation concerning the Landi and Held study was that for each air exposed treatment PPD (0, 24 hours, 2 weeks, 1 month, 4 months, 6 months, 9 months, and 12 months), 4 guinea pigs were potency tested for a total of 32 guinea pigs. Each group of 4 guinea pigs received injections of 4 different concentrations of their specific treatment PPD on one side of the back and the same 4 concentrations of a reference PPD on the other side of the back. This method is very similar to the 9 CFR method for potency testing in guinea pigs. Another difference is that the PPD utilized in

the Landi and Held study was prepared from a master batch of lyophilized PPD that had been produced years prior to their oxidation study.

In this current study, it was not only important to test potency by the approved guinea pig potency model but also in the natural host model. Cattle potency testing showed that there were no significant differences between all treatment bPPD responses and each bPPD caused a delayed-type hypersensitivity response that would classify each *Mycobacterium bovis* experimentally infected animal as a suspect; based on the CFT currently used by the USDA Bovine TB Eradication Program as a primary screening test and previously described in the Materials and Methods Section. For the CFT, the size of the response is inconsequential. If there is any response on the CFT, the animal is considered a suspect and retested using the CCT or Bovigam®.

The results obtained from the *in vitro* Bovigam® assay indicated that each treatment bPPD was able to effectively stimulate blood lymphocytes to produce IFN-γ to the same extent as the bPPD negative control reference. A reduced concentration of bPPD, avium PPD, and pokeweed mitogen was used in this study because of the known infection status of the 7 cattle utilized and the theory that if the treatment bPPDs had undergone changes due to oxidation, the reduced concentration would better reflect these changes.

The bPPD is primarily composed of protein; therefore an important aspect of this study was to determine if there were changes in the treatment bPPD groups after exposure to air. Protein determination indicated that there was no change from the starting protein

concentration of 1.08 mg/ml prior to air treatment and the final protein concentration for each treatment bPPD of 1.0 mg/ml to 1.1 mg/ml post air treatment.

Another important aspect of this study was to determine if there were changes to the pH of all treatment bPPDs when compared to the bPPD negative control reference. It is known that the pH of a protein solution is very important in regards to the stability of that solution. J.L. Cleland reported that in order to reduce factors that may contribute to chemical degradation (oxidation), a protein solution pH should be between 5 and 7 (Cleland 1993). A pH at this range may limit redox reactions that contribute to oxidation (Shacter 2000). The pH of each treatment bPPD remained unchanged at 7.32 to 7.33 when compared to the bPPD negative control reference.

Another necessary consideration to this study was to visually inspect all treatment bPPDs and compare to the bPPD negative control reference to determine if the bPPD protein solution had undergone any changes in response to air exposure. Since it has been shown that aggregate formation can occur in protein solutions and that these aggregates may cause adverse immunogenic effects, reduced efficacy, and that the protein solutions may appear turbid or physically separate from solution (precipitates) (Manning 2010), a visual inspection was performed. All treatment bPPDs, upon visual inspection, remained clear with no visual precipitate formation or color change.

The *in vivo* and *in vitro* testing as well as protein and pH determination, and visual inspection, provide evidence to indicate that bPPDs do not contain an environment that is

conducive to protein oxidation. The aim of this study was to determine if bPPD, dispensed in multi-use vials, will retain potency after exposure to air for up to 6 months after initial usage when compared to bPPD multi-use vials that remained unopened. There were no significant differences between each treatment bPPD when compared to the bPPD negative control reference, indicating that multi-use vials of bPPD retained potency for 6 months even after exposure to air. Results from this study may lend support to further consideration relating to shelf life of opened multi-use bPPD bottles allowing for extended use in the field, less waste of product, and the need for additional vials due to the 2-week discard date. This study may also benefit in vitro diagnostics that utilize bPPD for bovine TB testing.

FUTURE WORK

Little is still known of the exact mixture of proteins that are present in the bPPD. Characterization of these proteins would provide a better understanding of their immunological significance. Identification of the proteins would also allow for future examination of any chemical or physical instability that may affect the potency of the bPPD solution.

Another consideration for a future study would be to evaluate the potency of multi-use vials of bPPD that have been utilized in the field for bovine testing. This study (air exposure), as well as the study by Maes, *et al*. (temperature), focused on one single factor that could affect the potency of bPPD and each study was carried out under controlled environmental conditions. Testing for bovine tuberculosis using bPPDs does not happen under controlled environmental conditions. Under field conditions, multiple factors could potentially contribute to the decrease in bPPD potency. These factors may include: (1) fluctuations in temperature, (2) dirty field conditions that lead to contaminants on the outside of the vial that get delivered to the inside via insertion of the needle, (3) contaminants on the needle due to dropping or multiple use, or (4) contaminated air injected into the vials. These factors by themselves may not cause a reduction in potency however, these factors together may be of concern.

REFERENCES

Angus, R.D. (1978). Production of reference PPD tuberculins for veterinary use in the United States. *Journal of Biological Standardization*, Vol. 6, 221-227.

Berg, Jeremy, *et al*. (2002). *Biochemistry*. (5th ed.) New York, NY: W.H. Freeman and Company.

Bovigam® [Package insert]. Prionics USA, Inc., Lavista, NE; (Version 1.3).

Chi, E.Y. et al. Physical Stability of Proteins in Aqueous Solutions: Mechanism and Driving Forces in Nonnative Protein Aggregation. *Pharmaceutical Research*, Vol. 20, No. 9. September 2003.

Cleland, J.L. The Development of Stable Protein Formulations: A Close Look at Protein Aggregation, Deamidation, and Oxidation. *Critical Reviews in Therapeutic Drug Carrier Systems*, 10(4):307-377 (1993).

Doherty, M.L. *et al*. Effect of a recent injection of purified protein derivative on diagnostic tests for tuberculosis in cattle infected with *Mycobacterium bovis. Research in Veterinary Science* 1995, 58, 217-221.

Doherty, M.L. *et al*. A sequential study of the bovine tuberculin reaction. *Immunology* 1996 87 9-14.

Francis, J. *et al*. The Diagnosis of TB in Cattle with Special Reference to Bovine PPD Tuberculin. *Australian Veterinary Journal*, Vol. 49, May, 1973.

Gradmann, C. (2006). "Robert Koch and the white death: from TB to tuberculin." Microbes and Infection 8(1): 294-301.

Janeway, Charles A. *et al.* (2005). Immunobiology. (6th ed.). New York, NY: Garland Science Publishing.

Kaufmann, S. Nature Medicine Special Web Focus: TB (2000).

Konyha, L.D. (1977). Replacement of mammalian tuberculin with an M. bovis PPD tuberculin for field testing for TB. *Proceedings of Nineteenth Annual Meeting*, 1976, pp. 345-350. The American Association of Veterinary Laboratory Diagnosticians.

Landi, S. Preparation, purification, and stability of tuberculin. *Applied Microbiology*. 11: 408-412 (1963).

Landi, S. and Held, H. Physicochemical and Biological Studies on Various Preparations of Tuberculin Purified Protein Derivative. *Applied Microbiology*. 13: 132-139 (1965).

Landi, S. *et al*. Relationship Between the Molecular Weight of Tuberculoprotein and its Biological Potency. Proc. 11th int. Cong. Permanent Sect. microbial. Standard. Int. Ass. Microbial. Soc., Milan 1968; Progr. *Immunobiol. Standard*., vol. 4, pp. 512-520.

Landi, S. and Held, H. Interaction of Preservatives With Components Present In Tuberculin Purified Protein Derivative (PPD) Solutions. International Symnposium on Preservatives in Biological Products, San Francisco 1973. *Develp. Biol. Standard*., Vol. 24, pp. 167-179.

Landi, S. and Held, H. Stability of Dilute Solutions of Tuberculin Purified Protein Derivative. *Tubercle* 59 (1978) 121-133.

Landi, S. and Held, H. Preparation And Characterization of A Large Batch Of Tuberculin Purified Protein Derivative (PPD-CT68). *Ann. Sclavo*, 1980, 22:6, pp. 899-907.

Landi, S. and Held, H. Stability of a dilute solution of tuberculin purified protein derivative at extreme temperature. *Journal of Biological Standardization* (1981) 9, 195-199.

Landi, S. and Held, H. Effect of Oxidation On The Stability Of Tuberculin Purified Protein Derivative (PPD). International Symposium on BCG Vaccines and Tuberculins, Budapest, Hungary, 1983. *Develop. Biol. Standard*. Vol. 58, pp. 545-552.

Lochead, D.C. "Transmissibility of bovine TB to the human." *Proceedings of the 32nd Annual Meeting of the United States Livestock Sanitary Association*, pp. 526-547, Chicago, IL, USA, 1928.

Maes, M., Gimenez, J.F. *et al*. The stability of human, bovine and avian tuberculin purified protein derivative (PPD). *J Infect Dev Ctries* 2011; 5(11): 781-785.

Manning, M.C. *et al*. Stability of Protein Pharmaceuticals. *Pharmaceutical Research*, Vol. 6, No. 11, 1989.

Manning, M.C. *et al*. Stability of Protein Pharmaceuticals: An Update. *Pharmaceutical Research*, Vol. 27, No. 4, April 2010.

Marshall, C.J. "Progress in controlling bovine TB." *Journal of the American Medical Association*, vol. 80, pp. 625-633, 1932.

McMurry, J. (2000). Organic Chemistry. (5th ed.) Pacific Grove, CA: Brooks/Cole.

Merriam-Webster's Collegiate Dictionary. (2002). 10th edition. Springfield, MA: Merriam-Webster, Inc.

Meyer, R.M. *Official TB Program Tests and Their Use in the Field*. United States Department of Agriculture Basic TB Epidemiology Training Course. July 2004.

Miller, E.B. "Tuberculous cattle problem in the United States." *Historia Medicinae Veterinariae*, vol. 14, no. 1-2, pp. 1-64, 1989.

National Archives of the United States, Code of Federal Regulations, Title 9, Part 113.203, February 27, 1976A): 8471.

National Archives of the United States, Code of Federal Regulations, Title 9, Part 77.3, January 1, 2007. pp. 247-250.

National Archives of the United States, Code of Federal Regulations, Title 9 Part 113.26, January 1, 2007. p. 657.

National Archives of the United States, Code of Federal Regulations, Title 9 Part 113.38, January 1, 2007. p. 666.

National Archives of the United States, Code of Federal Regulations, Title 9 Part 113.409, January 1, 2007. pp. 767-769.

Palmer, M.V. *et al*. Aerosol delivery of virulent *Mycobacterium bovis* to cattle. *TB (Edinb)* 82:275-282, 2002.

Pritchard, D.G. (1988). "A century of bovine TB 1888-1988: Conquest and controversy." Journal of Comparative Pathology 99(4): 357-399.

Rennie, B. *et al*. Antibody response to a sterile filtered PPD tuberculin in M. bovis infected and M. bovis sensitized cattle. *BMC Veterinary Research* 2010, 6:50.

Shacter, E. Quantification and Significance of Protein Oxidation in Biological Samples. *Drug Metabolism Reviews*, 32(3&4), 307-326 (2000).

Siebert, F.B. (1934). The isolation and properties of the purified protein derivative of tuberculin. *Am Rev Tuberc* 30: 713-720.

Siebert, F.B. and Glenn, J.T. (1941) Tuberculin Purified Protein Derivative. *Am Rev Tuberc* 44: 9-25.

Siebert, F.B. (1944). The Chemistry of Tuberculin. *Chemical Reviews*, 34: 107-127.

Tubersol®. [Package Insert]. Tuberculin Purified Protein Derivative (Mantoux). Sanofi Pasteur Limited. Toronto, Ontario, Canada. January 2007.

United States Department of Agriculture. Bovine TB Eradication. *Uniform Methods and Rules*, January 1, 2005.

United States Department of Agriculture. Tuberculin Storage and Handling. *Veterinary Services Memorandum No. 552.11*. August 7, 2006.

Vaccination News. *Mantoux Test*. http://www.vaccinationnews.com/dailynews/May2001/TBTestInfo.htm. October 30, 2012.

WHO TB Research Office and Biophysics Laboratory of The University of Copenhagen. Effect Of Exposure Of Tuberculin To Light. *Bull. Wld Hlth Org*. 1955, 12: 179-188.

Wiker, H.G. et al. Immunochemical Characterization of the MPB70/80 and MPB83 Proteins of *Mycobacterium bovis*. *Infection and Immunity*, Apr. 1988, pp. 1445-1452.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the members of my committee: Dr. Jesse Hostetter, Iowa State University (ISU), Dr. W. Ray Waters, National Animal Disease Centers (NADC), and Dr. Walter Hyde, National Veterinary Services Laboratories (NVSL) for their patience, understanding, and support throughout this project.

I would like to thank my mentor and friend Mrs. Barbara Martin, NVSL, for her encouragement and faith in me. She believed in me when I did not believe in myself and stood firm when I needed someone to lean on.

My supervisor, Randy Capsel, NVSL, recognized the need for this project from the beginning. He ensured that I had all the necessary resources, including time, to undertake this study. His guidance and technical assistance as well as daily words of encouragement and review of this manuscript are greatly appreciated.

A special thanks to Dr. Derrick Rollins, my statistics professor at ISU, for his recommendations and review of this project's data.

The following individuals deserve a very special thank you: Mrs. Amber Peterson, NVSL, who performed all protein testing, Mrs. Renee Olsen, Center for Veterinary Biologics, who provided technical support and expertise for the guinea pig potency test, Mrs. Jessica Pollock, NADC, for her assistance with the Bovigam®, Ms. Janice Eifling, NADC for her outstanding journal support, the animal care staff at the National Centers for Animal Health for their care of the cattle and guinea pigs, and the animals themselves, for their ultimate sacrifice.